Lipocalin-2 is a Sensitive and Specific Marker of Bacterial Infection in Children

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Abstract
Introduction. Bacterial infection is the leading cause of death in children globally. Clinical algorithms to identify children who are likely to benefit from antimicrobial treatment remain suboptimal. Biomarkers that accurately identify serious bacterial infection (SBI) could improve diagnosis and clinical management. Lipocalin 2 (LCN2) and neutrophil collagenase (MMP-8) are neutrophil-derived biomarkers associated with bacterial infection. Methods: We evaluated LCN2 and MMP-8 as candidate biomarkers in 40 healthy controls and 151 febrile children categorised confirmed SBI, probable SBI, or viral infection. The diagnostic performance of LCN2 and MMP-8 to predict SBI was estimated by the area under the receiver operating characteristic curve (AUROC) and compared to the performance of C-reactive protein (CRP). Results. Plasma LCN2 and MMP-8 concentration were predictive of SBI. The AUROC (95% CI) for LCN2, MMP8 and CRP to predict SBI was 0.88 (0.82-0.94); 0.80 (0.72-0.87) and 0.89 (0.84-0.94), respectively. The diagnostic performance of LCN2 in combination with CRP was significantly superior to either marker alone: AUROC 0.92 (0.88-0.96). Conclusion. LCN2 is a sensitive and specific predictor of SBI in children which could be used to improve clinical management and antimicrobial stewardship. LCN2 should be further evaluated in prospective clinical studies.

Neutrophil gelatinase-associated Lipocalin (NGAL) | Severe bacterial infection| Lipocalin-2 | Sepsis | Biomarkers

Table 1. Demographic and clinical data of recruited subjects.

<table>
<thead>
<tr>
<th></th>
<th>Definite Bacterial</th>
<th>Probable Bacterial</th>
<th>Uncertain</th>
<th>Definite Viral</th>
<th>Healthy Control</th>
<th>P-value</th>
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<tr>
<td>Number of patients</td>
<td>42</td>
<td>42</td>
<td>62</td>
<td>42</td>
<td>42</td>
<td>N/A</td>
</tr>
<tr>
<td>Demographic data</td>
<td></td>
<td></td>
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<tr>
<td>Sex (male:female)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (years) median</td>
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<td>CRP (mg/l) median</td>
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<td>2.0 (0.00-0.00)</td>
<td>2.0 (0.00-0.00)</td>
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Methods. Patient recruitment. Between July 2009 and April 2012, we recruited acutely ill febrile children (age <17 years) presenting with illness of sufficient severity to warrant blood
tests. The study had approval of the St Mary’s Research Ethics Committee (REC 09/H0712/58). Written, informed consent was obtained.

Pathogen diagnosis. Bacterial diagnostics included culture of blood and (when clinically indicated) cerebrospinal and pleural fluid, and pneumococcal antigen detection in blood or urine. Respiratory or nasopharyngeal secretions were screened for viruses by nested PCR, including RV, coronavirus, adenovirus, parainfluenza 1-4, influenza A+B, bovivirus, metapneumovirus and rhinovirus.

Patient categorisation and selection. Patients were assigned to clinical categories corresponding to likelihood of infection after evaluation of all clinical, radiological and laboratory data (Figure 1). Patient categories were ‘Definite Bacterial’ (DB), ‘Probable Bacterial’ (PB), ‘Uncertain Bacterial or Viral’ (U), ‘Probable Viral’ (PV) or ‘Definite Viral’ (DV). DB children had microbiologically confirmed bacterial infection identified at a sterile-site, irrespective of the CRP value. As there were only 4 patients in the PV category, analysis was not carried out on this group. Controls (C) were recruited in-out-patients. After categorising, patients were prioritised for biomarker estimation based on the quantity of available plasma, and the chronological order of recruitment to give 5 groups as follows: 42 (DB), 34 (PB), 33 (U), 42 (V), 40 (C).

Biomarker measurement. Heparinised blood samples were kept at 4°C pending plasma recovery by centrifugation and storage at -80°C. LCN2 and MMP-8 were measured using commercially available immunoassays (RD Systems, UK) following manufacturer’s instructions, by laboratory personnel blinded to the diagnostic group of each sample. Presence of visible haemolysis and the number of previous plasma freeze-thaw cycles prior to EIA had no systematic effect on measurement. The coefficient of variation for these measurements was 8.26% (calculated from 28 samples with 2 replicates). CRP and creatinine values contemporary with research blood sampling were obtained from hospital data for 162 children (median and IQR: 39 and 34-50 µmol/L), and did not significantly differ between clinical groups (Kruskal-Wallis, P=0.36). The adjusted logistic regression analysis indicated that prediction of SBI by LCN2 was not affected by creatinine concentration (supplementary Table 1).

Combination of LCN2 and CRP to predict SBI. CRP was used as a benchmark to evaluate the performance of LCN2 to predict confirmed SBI in 191 patients with available CRP and LCN2 data. The AUROC of LCN2 and CRP were strongly correlated (r = 0.40, P<0.001). However, when both proteins were included into a logistic regression model, the combination was significantly superior to either marker alone (AUROC 0.92 [95% CI: 0.88-0.96]) (Figure 3 and Supplementary Table 1).

LCN2 and CRP in uncertain cohorts. Without a sensitive gold-standard test for detection of SBI, the majority of SBI is ‘missed’, therefore we correlated LCN2 and CRP biomarker concentrations with clinical suspicion of SBI. There was a stepwise increase in biomarker concentration with increasing suspicion of SBI: from C, DV, U, PB, to B (Table 1, Figure 4).

We derived cut-off values for LCN2 and CRP based on the concentration that showed the highest sensitivity and specificity to discriminate confirmed bacterial infection (DB) from other febrile patients (Supplementary Table 1). The sensitivity and specificity of LCN2 to predict DB were 83.3% and 82.5%. CRP had sensitivity and specificity of 85.7% and 83.8%. When the threshold value for LCN2 was applied to clinical categories corresponding to likelihood of infection, the majority of SBI was higher in the DB than DV group. LCN2 and MMP-8 are associated with SBI. Concentrations of plasma LCN2 and MMP-8 were lowest in controls, and increased stepwise with likelihood of SBI (Table 1). CRP, LCN2 and MMP-8 discriminated patients in the DB and DV or C groups (Figure 2a). We derived ROC curves for CRP, LCN2, MMP-8 for all 191 patients, and for neutrophil proportion and white cell counts in 175 patients with available values, based on the comparison of the DB group with the other febrile patients (combined PB, U and DV groups) (Figure 2b). LCN2 used as a continuous variable predicted confirmed SBI with an AUROC of 0.88 (95% CI: 0.82-0.94), matching the AUROC for CRP (0.89 [95% CI: 0.84-0.94]). MMP-8, neutrophil proportion and white cell count predicted confirmed SBI less well than LCN2 and CRP (AUROC 0.80, 0.71 and 0.69 respectively) (Figure 2b).
Children admitted with acute infection, sick enough to warrant blood tests, were recruited after presentation and before diagnostic studies were completed, at St Mary’s Hospital, London and University Hospital NHS Foundation Trust, Southampton, UK. Detailed clinical and laboratory data were recorded. Healthy controls were recruited in outpatients. After excluding patients with proven or possible inflammatory conditions or mycobacterial disease, two independent paediatric infectious disease clinicians with access to all clinical and diagnostic data categorised patients as follows. Children with a clinical syndrome in keeping with SBI (sepsis with shock or severe focal infection) were categorised as ‘Definite Bacterial’ (DB) only if pathogenic bacteria were detected at a usually sterile site such as blood or CSF, and not including surface swabs, endotracheal secretions or bronchoalveolar lavage samples; otherwise these patients were categorised as ‘Probable Bacterial’ (PB). Children with a clinical syndrome in keeping with viral infection, not displaying any bacterial features were categorised as ‘Definite Viral’ (DV) if a matching virus was identified, or otherwise as ‘Probable Viral’ (PV). Children without detected sterile-site bacteria and with inconclusive clinical features of viral or bacterial infection were classified as ‘Uncertains’ (U). We set a threshold of 60 mg/L for the maximum CRP as a minimum for inclusion into the PB group, or a maximum for inclusion in the PV and DV groups. Inclusion in the DB group was irrespective of CRP. Patients failing the CRP threshold were categorised as ‘Uncertain’ (U), alongside the other patients in this group with inconclusive clinical features. Controls (C) had no current or recent (previous two weeks) infectious symptoms or immunisations, and no identified or probable chronic infectious or inflammatory conditions. CRP: C-reactive protein. WCC: white cell count.

Discussion. This study shows that LCN2 is a sensitive and specific biomarker associated with SBI in children with febrile illness and has potential to guide antibiotic treatment decisions. The best diagnostic performance was achieved by combining LCN2 and CRP (AUROC of 0.92).

LCN2 is a 21-kD glycoprotein secreted by neutrophils, hepatocytes and renal tubular cells and it is usually found at low concentration (20 ng/mL) in biological fluids (8). The role of LCN2 in the innate defence against bacterial infection has been attributed to its ability to interfere with bacterial iron uptake through competition with the bacterial siderophile enterobactin (9, 10). The role of LCN2 as a potential marker of bacterial infection as described in previous studies (5, 11).
citonin is a promising biomarker of bacterial infection with allocate the patients to clinical groups (PB, U, DV). Procal-
parison would be confounded by the use of the CRP result to CRP in the groups with diagnostic uncertainty, as this com-
old). We did not compare the levels of LCN2 or MMP-8 to
and MMP-8 was proportional to the estimated likelihood of
We have shown that the biomarker concentration of LCN2
of this biomarker to guide antibiotic treatment is commonly
able as our predicted outcome to evaluate the diagnostic per-
The clinical group most likely to have SBI was defined by a
to capture the real world situation in which the majority of
prior antibiotic treatment at the time of diagnosis.
LCN2 has recently emerged as a biomarker of acute kidney
injury (7). We investigated if the association of LCN2 with SBI was confounded by impaired renal function. Adjusting
for creatinine concentration did not affect the di-
gnostic performance of LCN2 to predict SBI, which sug-
gests that neutrophils are the likely source of LCN2. The
diagnostic performance of LCN2 exceeded that of MPP-8, previously proposed as a paediatric sepsis biomarker (16).
In this study we have defined patient groups by a gradient from low- to high likelihood of SBI. This has the advantage to capture the real world situation in which the majority of patients have no definitively confirmed or excluded infection.
The clinical group most likely to have SBI was defined by a positive blood culture (gold standard) and we used this variable as our predicted outcome to evaluate the diagnostic performance of LCN2 and MPP-8. Thus, it was not possible to compare diagnostic value of LCN2 to the gold standard; instead, we used CRP for comparison as the measurement of this biomarker to guide antibiotic treatment is commonly used in secondary and tertiary care.
We have shown that the biomarker concentration of LCN2 and MPP-8 was proportional to the estimated likelihood of SBI, with unconfirmed patients tending to have intermediate levels (47% of PB group had LCN2 > 175.3 ng/mL threshold). We did not compare the levels of LCN2 or MPP-8 to CRP in the groups with diagnostic uncertainty, as this comparison would be confounded by the use of the CRP result to allocate the patients to clinical groups (PB, U, DV). Procalctinin is a promising biomarker of bacterial infection with kinetics comparable to those of LCN2 and it shown a modest advantage over CRP in the prediction of SBI (17), but we did not have these data for comparison.
Unless a biomarker is identified which discriminates SBI and non-SBI patients at non-overlapping concentrations, children with intermediate, ‘grey’ clinical markers of bacterial infection will remain as the group in whom the biomarkers are least effective, but most needed. The potential for LCN2 alone or in combination with CRP to improve the rapid ident-
ification of children with SBI at an early time, before culture results are available, could benefit clinical decision-making for children presenting with febrile illness, and improve anti-
tibiotic coverage in the easily missed minority of children with an SBI. The potential for LCN2 to improve antibiotic management decisions in febrile children should be evaluated in clinical trials.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ship: G0701885) and the NIHR (BRC/RCF: AC12/108).
We thank the patients and their families for taking part, Prof Saul Faust, Dr Sanjay Patel and Jenni McCorkell at the Uni-
versity of Southampton and Southampton University Hospi-
tal NHS Foundation Trust, and the clinicians and research teams for their support.

**Bibliography**

3. Peter J Gill, Michael J Goldacre, David Mant, Carl Heneghan, Anne Thomson, Valerie Sea-


Lipocalin 2 is a sensitive and specific marker of bacterial infection in children.

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Tables and figures
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<tr>
<th></th>
<th>Definite Bacterial</th>
<th>Probable Bacterial</th>
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<th>Definite Viral</th>
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<th>P value</th>
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<td>Sex: males (%)</td>
<td>18 (42.8)</td>
<td>14 (41.1)</td>
<td>19 (57.5)</td>
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<td>26 (65)</td>
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<td>29</td>
<td>35</td>
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<td>Caucasian (%)</td>
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<td>16 (53)</td>
<td>11 (38)</td>
<td>20 (57)</td>
<td>17 (49)</td>
<td>NS</td>
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<td>16 (48.4)</td>
<td>12 (28.5)</td>
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<td>4 (9.52)</td>
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<td>19 (55.8)</td>
<td>11 (33.3)</td>
<td>12 (28.5)</td>
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<td>2</td>
<td>0</td>
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<td>NS</td>
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<td>204 (120-226)</td>
<td>118.5 (69-142)</td>
<td>35 (10-87)</td>
<td>12 (1-23)</td>
<td>(0.56-1.96)</td>
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<td>LCN2 ng/mL: median (IQR)</td>
<td>348 (236-663)</td>
<td>157 (112-236)</td>
<td>143 (84-208)</td>
<td>103 (59.8-162)</td>
<td>72.5 (50-96)</td>
<td>&lt;0.0001</td>
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<td>MMP-8 ng/mL: median (IQR)</td>
<td>176 (50.1-302)</td>
<td>79.2 (34.3-150)</td>
<td>37.6 (12-101)</td>
<td>21.3 (9.56-74)</td>
<td>(4.37-28.4)</td>
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<td>WBC (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
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<td>75 (64-86)</td>
<td>71 (52-83)</td>
<td>62 (45-71)</td>
<td>44 (35-48)</td>
<td>&lt;0.0001</td>
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Table footnote: NS - not significant ($P > 0.05$); IQR – interquartile range; N/A - not applicable; PICU – paediatric intensive care unit; WBC – white cell count; \( ^a \)Not all patients had samples collected for virological investigation; \( ^b \) Some patients had both respiratory and non-respiratory viruses detected. The identified bacterium is listed for patients with Definite Bacterial infection. Two age comparisons were significant: the Probable Bacterial median age was higher than the Definite Viral group ($p < 0.01$), and the Definite Viral group were younger than the Controls ($p < 0.05$). The proportion of children with male sex or with Caucasian ethnicity (mode ethnicity) was not significantly different between groups. Severity of illness, as indicated by the number of children requiring PICU admission, inotrope use and ventilation, was highest in the Definite Bacterial group and lowest in the Definite Viral group. Deaths in each group were not significantly different.
Figure 1.

**Patient recruitment and sample collection n=394**
RNA collected in PAXgene tube from patients with febrile illness and from controls

**Categorisation of patients based on clinical data**
Patients grouped according to their clinical syndrome

- **BACTERIAL features**
  - Sepsis/suspected sepsis
  - Focal pyogenic infection
  - Focal pneumonia
  - Empyema
  - Meningitis (with polymorphs)
  - Bone infection
  - Urosepsis

- **VIRAL features**
  - Febrile illness without localising features
  - Flu-like illness
  - Respiratory illness without consolidation or empyema
  - Meningitis (with lymphocytes)

- **CONTROL**
  - No recent infection
  - Not recently immunised

- **OTHER**
  - Not infection or other infection (TB, fungal)

**Review supporting microbiological & clinical information**
Bacterial and viral tests, radiology, blood tests, disease severity & supportive care

- **Sterile-site pathogenic bacteria identified, and match syndrome**
  - Definite Bacterial n=58
  - Probable Bacterial n=44
  - Uncertain Bacterial or Viral n=103

- **Bacterial syndrome, but no bacteria identified**
  - Definite Viral n=78
  - Probable Viral n=4

- **Inconclusive features OR microbiology does not fit syndrome**
  - Uncertain Viral n=33

- **Virus identified, and matching syndrome**
  - Definite Viral (DV) n=42

- **Viral syndrome, but no virus identified**
  - Control n=67
  - Excluded n=40

**Selection of plasma samples for assay**
Determined by chronology of recruitment, number of available plasma aliquots

- **Definite Bacterial (DB) n=42**
- **Probable Bacterial (PB) n=34**
- **Uncertain (U) n=33**
- **Definite Viral (DV) n=42**
- **Control (C) n=40**
Figure 2.

(A)

(B)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>N</th>
<th>Area</th>
<th>(95% CI)</th>
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<tr>
<td>LCN2 (ng/mL)</td>
<td>191</td>
<td>0.88</td>
<td>(0.82 - 0.94)</td>
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<tr>
<td>MMP-8 (ng/mL)</td>
<td>191</td>
<td>0.80</td>
<td>(0.72 - 0.87)</td>
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<tr>
<td>CRP (µg/mL)</td>
<td>191</td>
<td>0.89</td>
<td>(0.84 - 0.94)</td>
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<tr>
<td>Neutrophil (%)</td>
<td>175</td>
<td>0.71</td>
<td>(0.61 - 0.80)</td>
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<tr>
<td>White cell count (10⁶/L)</td>
<td>175</td>
<td>0.69</td>
<td>(0.59 - 0.79)</td>
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*P = 0.0009
Figure 3.

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<td>LCN2</td>
<td>191</td>
<td>0.88</td>
<td>(0.82-0.94)</td>
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<tr>
<td>CRP</td>
<td>191</td>
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<td>(0.84-0.94)</td>
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<td>LCN2+CRP</td>
<td>191</td>
<td>0.92</td>
<td>(0.88-0.96)</td>
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P<0.001
Figure 4.
Supplementary Material

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Contents

Supplementary Table 1
Sensitivity and specificity of LCN2, CRP and combined LCN2 and CRP

Supplementary Table 2
Association of LCN2 with confirmed bacterial infection in a univariate logistic regression model adjusted for creatinine concentration
Supplementary Table 1 – sensitivity and specificity of LCN2, CRP and combined LCN2 and CRP

<table>
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<tr>
<th>cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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<td>82.5</td>
<td>57</td>
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<tr>
<td>CRP (ug/mL)</td>
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<td>85.7</td>
<td>83.8</td>
<td>60</td>
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<td>LCN2 + CRP</td>
<td>175.3/69</td>
<td>90.4</td>
<td>86.5</td>
<td>66</td>
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</table>

PPV positive predictive value, NPV: negative predictive value

Identification of the cut-off value with the highest balance of sensitivity and specificity to distinguish between definite bacterial infection (DB vs. non-DB) for LCN2, CRP and combined LCN2 and CRP. The combination of LCN2 and CRP had the highest sensitivity and specificity corresponded to values above the 7th decile of LCN2 and CRP concentration.
Supplementary Table 2 – Association of LCN2 with definite bacterial infection: logistic regression model adjusted for serum creatinine concentration. Serum creatinine was available for 161 patients.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Confirmed bacterial infection</th>
<th>OR (95%CI)</th>
<th>[P value]</th>
<th>N of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate logistic regression</td>
<td>crude</td>
<td>adjusted*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCN2 (ng/mL)</td>
<td>1.006 (1.003-1.009)</td>
<td>[&lt;0.001]</td>
<td>N=191</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.005 (1.002-1.008)</td>
<td>[&lt;0.001]</td>
<td>N=161</td>
<td></td>
</tr>
</tbody>
</table>

As LCN2 levels increase with renal damage [1], we determined whether renal impairment influenced performance. Creatinine levels contemporaneous with research bloods were available for 162 children (median and IQR: 39 and 34-50 µmol/L), and did not significantly differ between clinical groups (Kruskal-Wallis, \( P=0.36 \)). The adjusted logistic regression analysis indicated that prediction of DB by LCN2 was not affected by creatinine concentration.

References